Immunity, Vol. 21, 67–80, July, 2004, Copyright ©2004 by Cell Press

Signaling through Itk Promotes T Helper 2 Differentiation via Negative Regulation of T-bet

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Summary

The Tec family tyrosine kinase, Itk, is critical for PLC-γ1 activation downstream of the TCR. Studies of Itk$^{-/-}$ mice have demonstrated a requirement for Itk in Th2 cytokine production and protective immunity to parasitic infections. Here we address the mechanism by which Itk regulates Th2 differentiation. We find that naive Itk$^{-/-}$ CD4^{+} T cells respond normally to cytokine skewing signals and can differentiate efficiently into either Th1 or Th2 lineage cells. In the absence of skewing cytokines, wild-type CD4^{+} T cells stimulated with low-avidity ligands preferentially express GATA-3 mRNA and differentiate into Th2 cells. Under these same stimulation conditions, Itk$^{-/-}$ T cells produce large amounts of T-bet mRNA and differentiate into IFN-γ-producing cells. Furthermore, Itk is upregulated during Th2 differentiation, while Rlk, a related Tec kinase, disappears rapidly from differentiating Th2 cells. Together, these findings provide a molecular explanation for the essential role of Itk in Th2 differentiation.

Introduction

Triggering of the T cell antigen receptor (TCR) is one of the hallmarks of the adaptive arm of the immune system. Following this highly specific interaction, a complex series of biochemical events takes place within the T cell, culminating in the activation of specific genes. Over the past several years, there has been increasing evidence, both in vitro and in vivo, demonstrating the importance of the Tec family tyrosine kinase, Itk, in the generation of critical CD4^{+} T cell effector functions.

Expressed predominantly in T cells and mast cells, Itk is activated in response to antigen receptor stimulation. Biochemical studies have indicated that signaling through the TCR leads to Itk recruitment to a multimolecular complex that includes SLP-76, LAT, Gads, Grb2, and PLC-γ1, providing a platform for Itk to interact with and phosphorylate PLC-γ1 (Lucas et al., 2003; Miller and Berg, 2002b). In support of this model, TCR stimulation of Itk$^{-/-}$ CD4^{+} T cells results in substantially impaired PLC-γ1 tyrosine phosphorylation and activation, intracellular calcium mobilization, and MAP kinase activation, as well as NFATc nuclear translocation (Fowell et al., 1999; Liu et al., 1998; Schaeffer et al., 1999, 2001). As a consequence, the transcription of numerous cytokine and effector genes, such as IL-2, IL-4, IFN-γ, and FasL are all reduced in stimulated Itk$^{-/-}$ CD4^{+} T cells (Fowell et al., 1999; Liu et al., 1998; Miller and Berg, 2002a; Schaeffer et al., 1999, 2001). Overall, these data have indicated that TCR signaling in the absence of Itk is greatly diminished, but not entirely abolished.

Interest in the role of Itk in CD4^{+} T cell effector function was heightened by intriguing observations demonstrating that Itk$^{-/-}$ mice have impaired responses to pathogenic infection. For instance, in response to *T. gondii*, a pathogen that normally elicits a protective Th1 response, Itk-deficient mice succumb to the infection with a mean survival time of 69 days (Schaeffer et al., 1999). In contrast, in response to *L. major*, a pathogen that normally elicits a protective Th1 response in C57BL/6 mice and a nonprotective Th2 response in Balb/c mice, Itk-deficient mice on both genetic backgrounds mounted a protective Th1 response (Fowell et al., 1999). However, in response to the nematode *N. brasiliensis*, which elicits a protective Th2 response in Balb/c mice, Itk$^{-/-}$ Balb/c mice were unable to clear the infection. Further in vitro data demonstrated that an inability to translocate NFATc to the nucleus following TCR activation contributed to the observed impairment in IL-4 gene expression in Itk$^{-/-}$ cells, as well as to the failure of these cells to differentiate into Th2 effector cells in vivo (Fowell et al., 1999). In addition, a very recent study observed an impairment in basal chromatin modification at the IL-4 locus in Itk$^{-/-}$ cells activated in Th2 conditions (Grogan et al., 2003). Data from Schaeffer et al. further supported these observations by showing that, upon challenge with the helminth *S. mansoni*, Itk$^{-/-}$ mice generate a Th1 effector response to this pathogen instead of the Th2 response normally observed in wild-type mice (Schaeffer et al., 2001). Finally, recent work from Mueller and August examined an allergic asthma response after priming with the antigen ovalbumin and found that Itk$^{-/-}$ mice did not generate this allergic response (Mueller and August, 2003). Collectively, these data demonstrate that mice lacking Itk exhibit a selective and profound impairment in generating Th2-polarized CD4^{+} T cell responses.

The ability of naive CD4^{+} T cells to differentiate into Th1 or Th2 effector cells is regulated by a number of factors, the most important of which is the cytokine milieu during T cell activation. Although many cell types, such as dendritic cells, natural killer cells, and mast cells, can serve as a source of effector cytokines that influence T helper cell differentiation, the initial source of these cytokines in vivo following pathogenic infection is not always apparent. One hypothesis is that the T cells themselves produce cytokines that affect their own fate. In this regard, in vitro studies have demonstrated that varying the strength of TCR signaling by altering antigen concentration or potency can strongly influence T helper cell polarization (reviewed in Leitenberg and Bottomly, 1999).

In this study we sought to clarify the role of Itk in T helper cell differentiation. To this end, we established

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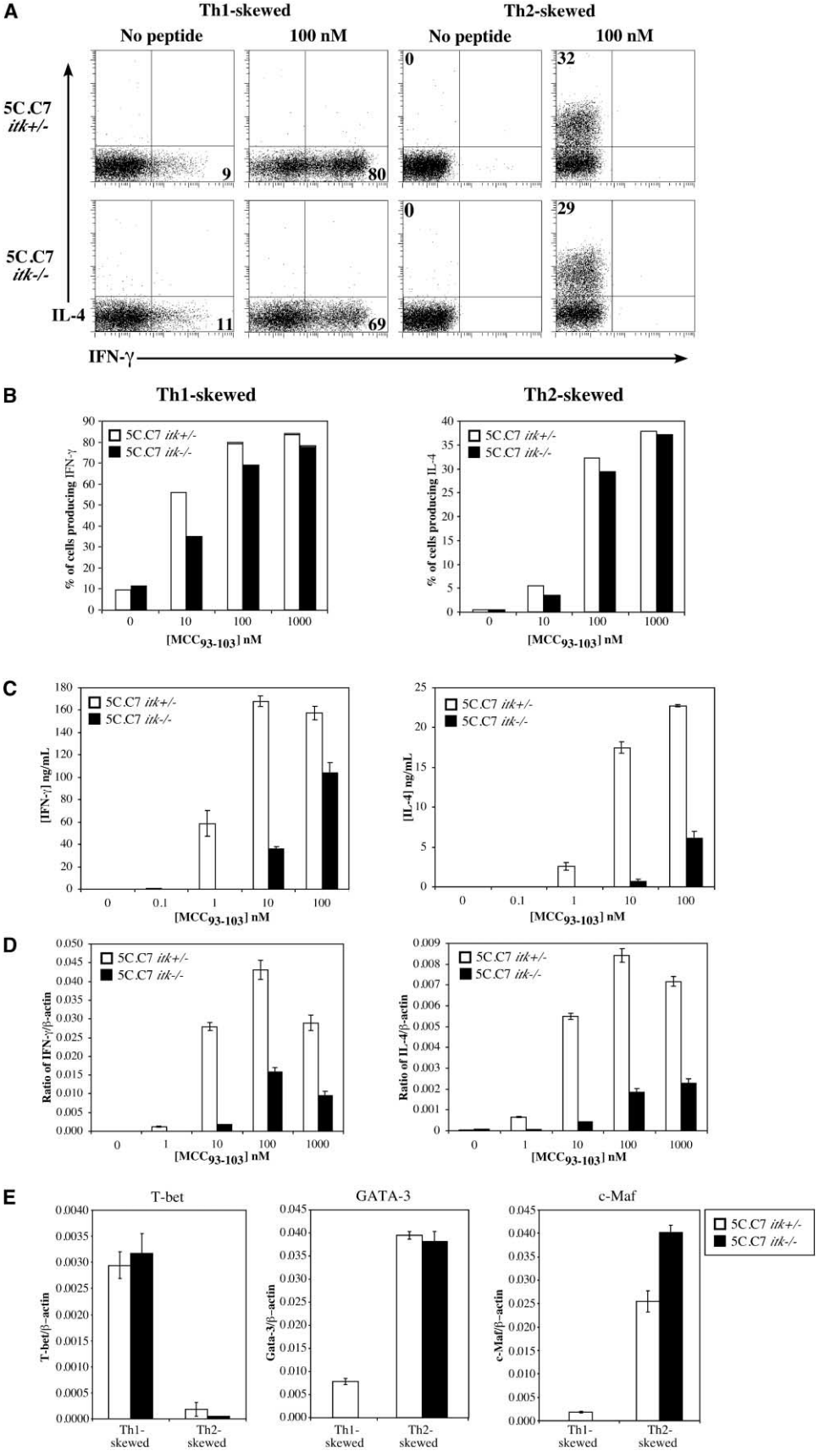
A
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Th2-skewed
No peptide
100 nM
No peptide
100 nM
5C.C7 itk+/-
5C.C7 itk-/-
IL-4
IFN-γ
9
80
0
32
11
69
0
29
B
Th1-skewed
Th2-skewed
5C.C7 itk+/-
5C.C7 itk-/-
% of cells producing IFN-γ
% of cells producing IL-4
[MCC93-103] nM
C
[IFN-γ] ng/mL
[IL-4] ng/mL
D
Ratio of IFN-γ/β-actin
Ratio of IL-4/β-actin
E
T-bet
GATA-3
c-Maf
T-bet/β-actin
Gata-3/β-actin
c-Maf/β-actin
Th1-skewed
Th2-skewed

an in vitro system to examine T helper cell activation and differentiation in the absence of Itk. These studies established that Itk is required for Th2 differentiation due to two factors: first, the rapid loss of Rlk in differentiating Th2 cells, and, second, a role in the negative regulation of T-bet expression.

Results

Itk$^{-/-}$ CD4^{+} T Cells Can Efficiently Differentiate into Polarized Th1 and Th2 Cells

Previous in vitro studies examining effector cytokine production by Itk$^{-/-}$ CD4^{+} T cells have indicated that these cells show dramatic defects in effector cytokine production. On the basis of ELISA data measuring cytokine secretion following restimulation, Itk$^{-/-}$ CD4^{+} T cells were found to be severely impaired in IL-4 secretion, with a less profound defect in IFN-γ secretion (Fowell et al., 1999; Schaeffer et al., 2001). However, these studies did not distinguish between defective T helper cell differentiation per se versus a selective defect in effector cytokine gene expression following TCR restimulation. To examine this issue, we established an in vitro assay using homogeneous populations of naive CD4^{+} T cells. To accomplish this, Itk$^{-/-}$ and Itk$^{+/-}$ mice were crossed to the 5C.C7 TCR transgenic line on a RAG$^{-/-}$ background. Naive CD4^{+} T cells were isolated from these mice and stimulated in vitro with CH27 B lymphoma cells as antigen-presenting-cells (APCs) plus the cognate antigen, a peptide derived from moth cytochrome c ($MCC_{93\text{-}103}$).

In our initial experiments, T cells were stimulated in both Th1-skewing (rIL-12 + anti-IL4 antibody) and Th2-skewing (rIL-4 + anti-IFN-γ antibody) conditions for 3 days in the presence of excess rIL-2. Following this 3 day culture period, cells were restimulated with APCs and varying doses of the $MCC_{93\text{-}103}$ peptide for 6 hr and assayed for IFN-γ and IL-4 by intracellular cytokine staining. Figure 1A shows raw data obtained from this analysis, indicating that, as expected, cells stimulated in Th1-polarizing conditions produce IFN-γ and not IL-4, whereas cells stimulated in Th2-polarizing conditions produce IL-4 and not IFN-γ. Figure 1B shows a bar graph indicating the percentage of cells that are producing either IFN-γ or IL-4. From these data, it is clear that both Itk$^{+/-}$ and Itk$^{-/-}$ cells are able to produce the appropriate effector cytokine after stimulation in either Th1- and Th2-polarizing conditions; furthermore, in the absence of Itk there is only a slight decrease in the percentage of cells that have differentiated into effector cells of each lineage. However, examination of the mean fluorescence intensity of IFN-γ and IL-4 staining indicates that, on a per cell basis, Itk$^{-/-}$ T cells produce less of the respective cytokine than wild-type T cells (data not shown). A similar observation has been reported for IL-2 production by Itk$^{-/-}$ CD4^{+} T cells (Wilcox and Berg, 2003). Overall, these data demonstrate that after stimulation in the presence of skewing cytokines, nearly equivalent percentages of Itk-deficient cells differentiate into both Th1 and Th2 effector cells. We have consistently observed, as others have (Openshaw et al., 1995), that upon stimulation of CD4^{+} T cells in either Th1- or Th2-skewing conditions, a greater proportion of cells differentiate into Th1 effectors than Th2 effectors.

To determine whether the amount of effector cytokines produced by Itk-deficient cells is indeed decreased compared to wild-type T cells, we restimulated both Th1- and Th2-skewed cells from Itk$^{+/-}$ and Itk$^{-/-}$ cultures for 24 hr with APCs and varying concentrations of the $MCC_{93\text{-}103}$ peptide, and then assayed for IFN-γ and IL-4 in the supernatants by ELISA. As shown in Figure 1C, Itk$^{-/-}$ Th1 cells secrete 1.5- to 200-fold less IFN-γ than Itk$^{+/-}$ T cells. Likewise, Th2 cells lacking Itk secrete 3.5- to 130-fold less IL-4 than control T cells. In addition, we found similar reductions in IL-5 and IL-10 secretion by Itk-deficient Th2 cells (data not shown). These results are comparable to data reported by Fowell et al. and Schaeffer et al. following stimulation of Itk$^{-/-}$ cells by anti-CD3 antibody crosslinking (Fowell et al., 1999; Schaeffer et al., 2001). However, it is apparent from the data presented here that the magnitude of the deficiency observed varies significantly depending on the dose of antigen used to restimulate the cells. This is consistent with many of our previous studies indicating that the signaling deficiencies observed in the absence of Itk are greatly exacerbated by suboptimal TCR stimulation (Miller and Berg, 2002a; Wilcox and Berg, 2003). This finding may account for discrepancies in effector cytokine production profiles of Itk$^{-/-}$ T cells reported previously (Fowell et al., 1999; Schaeffer et al., 2001).

Thus far our data indicate that Itk$^{-/-}$ CD4^{+} T cells can differentiate efficiently into both Th1 and Th2 effector cells but that these differentiated cells are poor producers of their respective effector cytokines. Based on the role of Itk in PLC-γ1 activation downstream of the TCR, it seemed likely that the cytokine production defect re-

Figure 1. Itk$^{-/-}$ CD4^{+} T Cells Can Efficiently Differentiate into Polarized Th1 and Th2 Cells

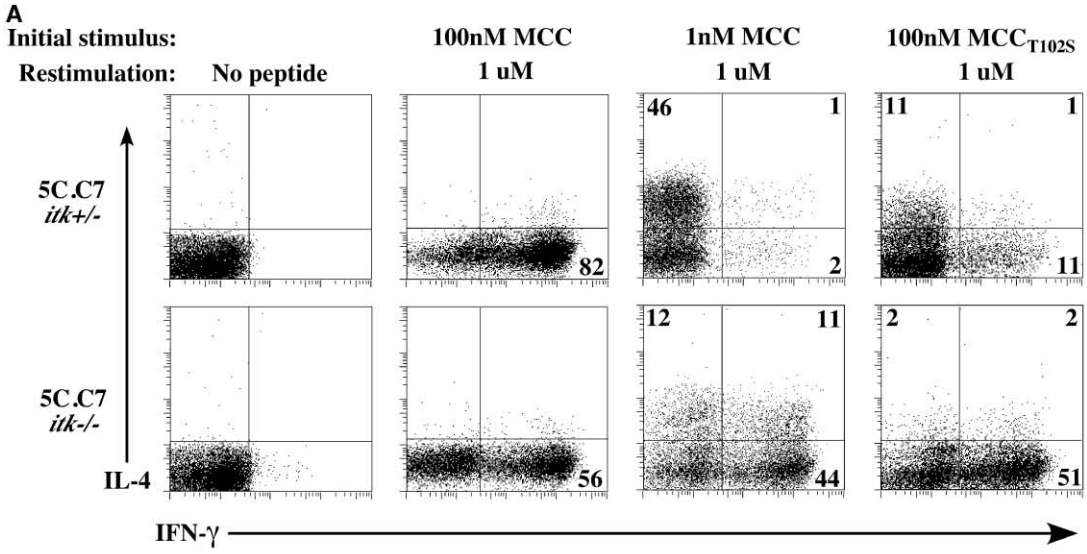
Purified CD4^{+} T cells from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice were stimulated in vitro with APCs and peptide in Th1- or Th2-skewing conditions.

(A) Following 3 days of culture, cells were restimulated with fresh APCs and $MCC_{93\text{-}103}$ peptide for 6 hr, stained with anti-CD4-Cy and anti-Vα11-FITC, fixed, permeabilized, and stained intracellularly with anti-IL-4-PE and anti-IFN-γ-APC. Ten thousand CD4^{+} Vα11^{+} events were collected on a flow cytometer. An example of raw data is shown.

(B) Intracellular cytokine staining data from the experiment shown in (A) in bar graph format, depicting the percentage of cells that are producing IFN-γ or IL-4, in the Th1- or Th2-skewed cultures, respectively, in response to varying doses of $MCC_{93\text{-}103}$.

(C) Following 7 days in culture, cells were restimulated for 24 hr with fresh APCs and varying doses of $MCC_{93\text{-}103}$. Supernatants were analyzed for the production of IFN-γ and IL-4, respectively, by ELISA. Error bars indicate SD of values obtained from stimulations performed in triplicate.

(D and E) Cells were restimulated for 6 hr on day 7 with fresh APCs and varying doses of $MCC_{93\text{-}103}$. Following stimulation, RNA was isolated and 1 μg was reverse transcribed into cDNA and subjected to real-time quantitative PCR analysis for β-actin, IFN-γ, and IL-4 (D), or T-bet, GATA-3, and c-Maf (E). The y axis values represent the respective transcript normalized to the β-actin values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions.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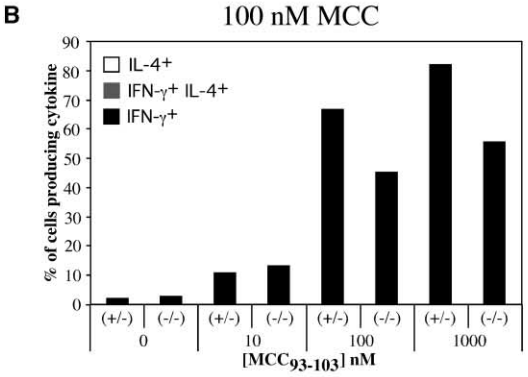
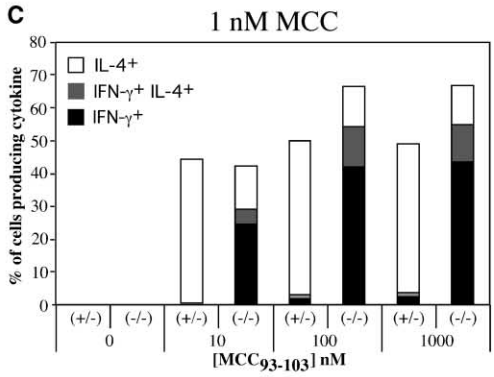
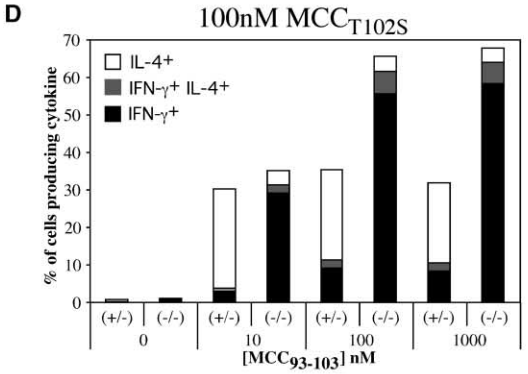
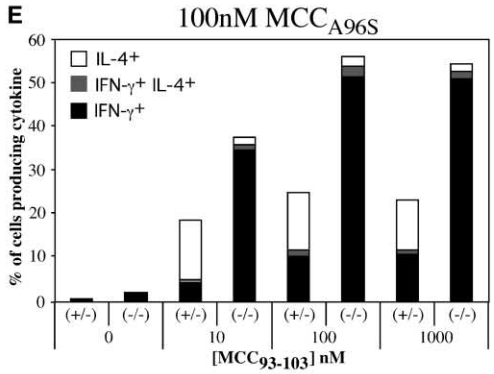
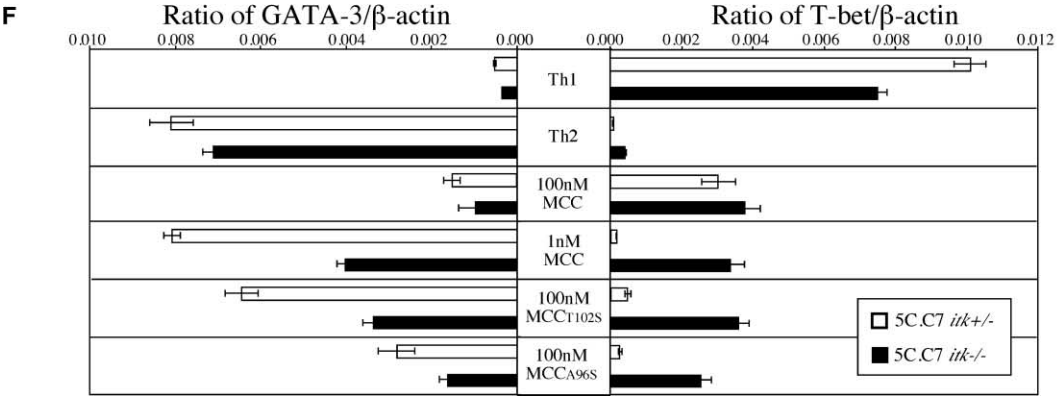
100nM MCC
1nM MCC
100nM MCC$_{T102S}$
Restimulation:
No peptide
1 uM
1 uM
1 uM
5C.C7
itk+/-
5C.C7
itk-/-
IL-4
IFN-γ
46
1
82
2
11
1
11
12
11
56
44
2
2
51


B
100 nM MCC
IL-4+
IFN-γ+ IL-4+
IFN-γ+
% of cells producing cytokine
(+/-)
(-/-)
0
10
100
1000
[MCC$_{93-103}$] nM


C
1 nM MCC
IL-4+
IFN-γ+ IL-4+
IFN-γ+
% of cells producing cytokine
(+/-)
(-/-)
0
10
100
1000
[MCC$_{93-103}$] nM


D
100nM MCC$_{T102S}$
IL-4+
IFN-γ+ IL-4+
IFN-γ+
% of cells producing cytokine
(+/-)
(-/-)
0
10
100
1000
[MCC$_{93-103}$] nM


E
100nM MCC$_{A96S}$
IL-4+
IFN-γ+ IL-4+
IFN-γ+
% of cells producing cytokine
(+/-)
(-/-)
0
10
100
1000
[MCC$_{93-103}$] nM


F
Ratio of GATA-3/β-actin
Ratio of T-bet/β-actin
0.010
0.008
0.006
0.004
0.002
0.000
0.000
0.002
0.004
0.006
0.008
0.010
0.012
Th1
Th2
100nM MCC
1nM MCC
100nM MCC$_{T102S}$
100nM MCC$_{A96S}$
5C.C7 itk+/-
5C.C7 itk-/-

sulted from impaired transcriptional activation of the IFN-γ and IL-4 genes, respectively, in differentiated Itk$^{-/-}$ T cells. To test for steady-state mRNA levels of IFN-γ and IL-4, we performed real-time quantitative RT-PCR on RNA prepared following a 6 hr restimulation of Th1- and Th2-skewed cells. For these experiments, Itk$^{+/-}$ and Itk$^{-/-}$ cells were stimulated with APCs and varying concentrations of the MCC$_{93\text{-}103}$ peptide. As shown in Figure 1D, these data precisely parallel the cytokine secretion data, showing greatly reduced transcripts for both IFN-γ and IL-4 in Itk$^{-/-}$ effector cells following TCR stimulation. These findings confirm that reduced cytokine secretion by Itk$^{-/-}$ T cells is a consequence of reduced transcription of these cytokine genes in differentiated Itk$^{-/-}$ effector CD4$^+$ T cells.

The data presented above strongly suggest that Itk$^{-/-}$ CD4$^+$ T cells have no intrinsic defect in their ability to respond to extrinsic cytokine signals by differentiating into the appropriate lineage of helper T cell. To assess this issue further, we examined mRNA levels for T-bet and GATA-3, two transcription factors that are the master regulators of Th1 and Th2 differentiation, respectively (Grogan and Locksley, 2002; Murphy and Reiner, 2002). As shown in Figure 1E, Itk$^{-/-}$ Th1 and Th2 cells express comparable levels of T-bet and GATA-3, respectively, compared to Itk$^{+/-}$ cells. In addition, we examined the mRNA levels for another transcription factor, c-Maf, that is essential for *trans*-activation of the IL-4 promoter in Th2 cells (Kim et al., 1999). As can be seen in Figure 1E, Itk$^{-/-}$ Th2 cells show no reduction in c-Maf mRNA levels compared to control Th2 cells. Taken together, these data demonstrate that, given exogenous cytokines, Itk-deficient CD4$^+$ T cells are fully capable of differentiating into both Th1 and Th2 cells, as evidenced by their normal frequency of effector cytokine producers as well as by their expression of the signature Th1 or Th2 transcription factors. Nonetheless, these cells are impaired in their ability to produce IFN-γ and IL-4 on a per cell basis compared to control T cells. As Itk$^{-/-}$ Th1 cells have wild-type levels of T-bet, and Itk$^{-/-}$ Th2 cells have normal levels of GATA-3 and c-Maf, it is likely that their defects in effector cytokine production reflect reduced activation of additional factors required for the transcription of these cytokine genes.

Low-Avidity TCR Stimulation Promotes Th2 Differentiation by Itk$^{+/-}$, but Not by Itk$^{-/-}$, CD4$^+$ T Cells

As described above, previous infectious disease studies examining immune responses of Itk$^{-/-}$ mice to *L. major*, *S. mansoni*, and *N. brasiliensis* had indicated that Itk$^{-/-}$ T cells were selectively impaired in generating protective Th2 responses. To determine whether these in vivo findings could result from intrinsic defects in Itk$^{-/-}$ CD4$^+$ T cell responses, we sought to establish in vitro conditions to test this hypothesis. To address this issue, we required a system that preferentially leads to Th2 differentiation by wild-type CD4$^+$ T cells in the absence of overt skewing by cytokines and blocking antibodies. Since our Itk$^{-/-}$ mouse line is on a C57Bl/10 background, we knew that straightforward in vitro stimulation of naive CD4$^+$ T cells with MCC peptide plus APCs would lead to almost exclusive Th1 polarization. However, a number of previous studies have demonstrated that stimulation of naive CD4$^+$ T cells with high- versus low-avidity TCR engagement can differentially promote Th1 versus Th2 differentiation, respectively, in the absence of exogenous cytokines (Leitenberg and Bottomly, 1999).

As shown in Figure 2, this phenomenon holds true for naive T cells purified from Itk$^{+/-}$ 5C.C7 TCR transgenic RAG$^{-/-}$ mice. For these experiments, T cells were initially stimulated with APCs plus a high (100 nM) versus a low (1 nM) concentration of MCC$_{93\text{-}103}$. T cells were also stimulated with high concentrations of two APLs, MCC$_{T102S}$ and MCC$_{A96S}$, that have previously been shown to be recognized with lower affinities and to induce weaker responses by 5C.C7$^+$ T cells (Lyons et al., 1996; Rabinowitz et al., 1996). After 3 days of culture in the absence of any additional cytokines or blocking antibodies, T cells were restimulated with fresh APCs and varying concentrations of the normal MCC$_{93\text{-}103}$ peptide for 6 hr. Cells were then stained intracellularly for IFN-γ and IL-4 (as in Figure 1A) and analyzed by flow cytometry. As can be seen in the raw data shown in Figure 2A and summarized in bar graph format in Figures 2B–2E, Itk$^{+/-}$ T cells differentiate predominantly into IFN-γ-producing cells following stimulation with a high concentration of MCC$_{93\text{-}103}$ but become IL-4-secreting cells when initially stimulated with a low concentration of MCC$_{93\text{-}103}$. Both of the APLs induce a mixed response from Itk$^{+/-}$ cells, with cultures displaying proportions of both IFN-γ- and IL-4-producing cells. Interestingly, when 5C.C7 TCR transgenic RAG$^{-/-}$ Itk$^{-/-}$ CD4$^+$ T cells were stimulated with these varying peptides and peptide concentrations, and then restimulated to test for T helper cell differentiation, all of the stimulation conditions induced Itk$^{-/-}$ T cells to become IFN-γ-producing cells (Figures 2A–2E). Collectively, these data demonstrate that, upon stimulation in conditions that induce wild-type T cells to differentiate into the Th2 lineage, Itk-deficient T cells are impaired in their ability to differentiate into this lineage and instead differentiate into Th1 cells. In some cases, the cultures of Itk-deficient T cells contain a greater percentage of cells that have differentiated, as

Figure 2. Low-Avidity TCR Stimulation Promotes Th2 Differentiation by Itk$^{+/-}$, but Not by Itk$^{-/-}$, CD4$^+$ T Cells

Purified CD4$^+$ T cells from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice were stimulated in vitro with APCs and either 100 or 1 nM of MCC$_{93\text{-}103}$, 100 nM of MCC$_{T102S}$, or 100 nM of MCC$_{A96S}$. Following 3 days in culture, cells were restimulated for 6 hr and assayed for IFN-γ and IL-4 production by intracellular cytokine staining as described in Figure 1.

(A) An example of raw data showing cytokine staining profiles.

(B–E) The percentages of cells that produce either IFN-γ, IL-4, or both are depicted in bar graph formats corresponding to the initial stimulus: 100 nM MCC$_{93\text{-}103}$ (B), 1 nM MCC$_{93\text{-}103}$ (C), 100 nM MCC$_{T102S}$ (D), or 100 nM MCC$_{A96S}$ (E).

(F) RNA was isolated from cells on day 3, transcribed into cDNA, and subjected to real-time quantitative PCR analysis for β-actin, T-bet, and GATA-3. The y axis values represent the respective transcript normalized to the β-actin values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions.

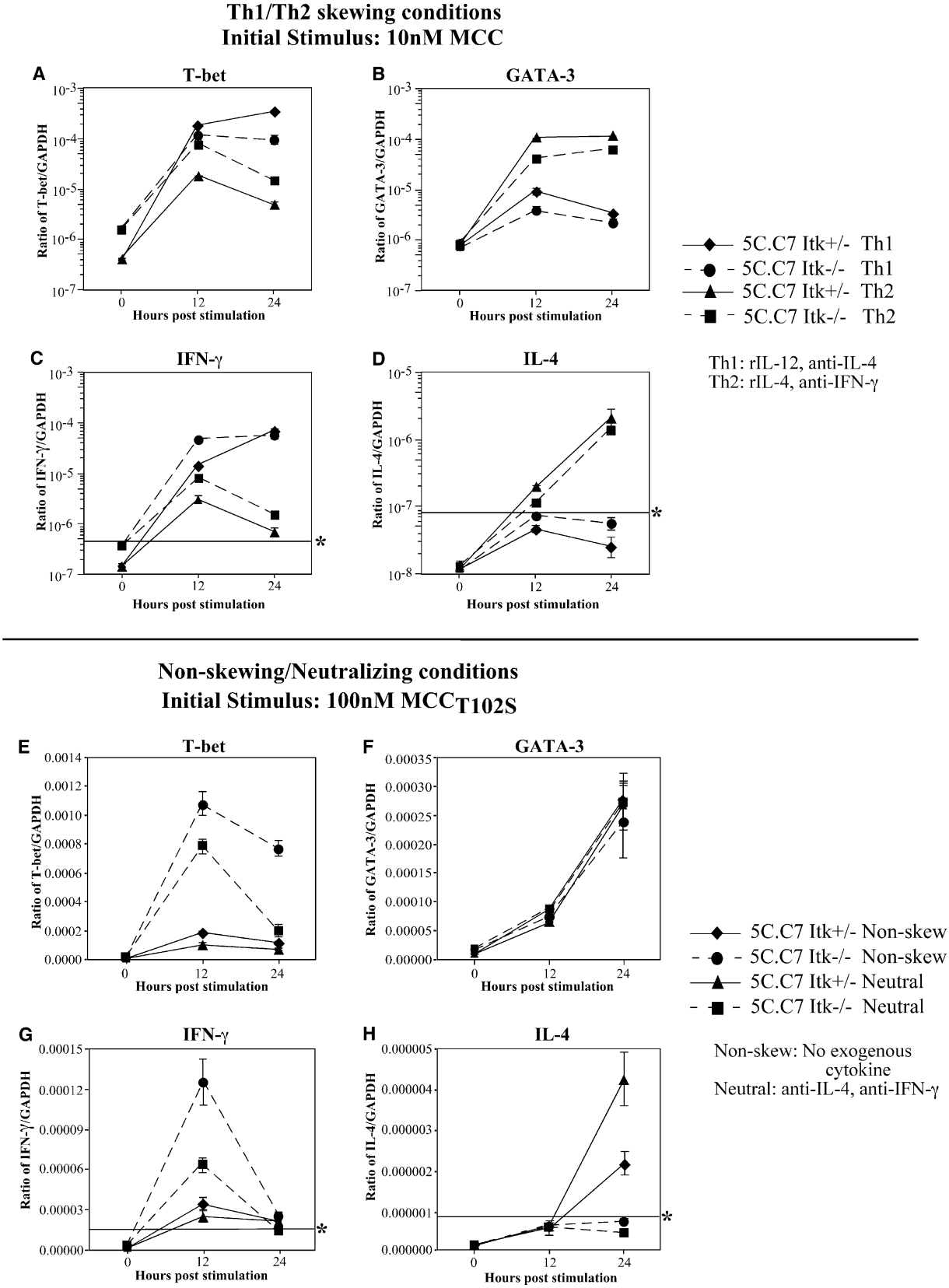


Figure 3. T-bet mRNA Expression Is Aberrantly Regulated by TCR Signals in the Absence of Itk

Purified CD4$^+$ T cells from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice were stimulated in vitro with APCs and MCC$_{93-103}$ in Th1- or Th2-skewing conditions (A–D) or stimulated with APCs and 100 nM of MCC$_{T102S}$ (E–H) in either nonskewing (no exogenous cytokine) or

measured by the total percent of cells producing effector cytokines (see Figures 2C–2E); this interesting phenomenon may be a consequence of the fact that Th1 cells are more efficiently generated in vitro than Th2 cells (see Figure 1B).

To further assess the differentiation status of cells stimulated in the various conditions, we examined the levels of GATA-3 and T-bet mRNAs 3 days poststimulation in Th1- and Th2-skewing conditions, as well as in the various nonskewing conditions shown in Figures 2B–2D. As shown in Figure 2F, $Itk^{+/-}$ as well as $Itk^{-/-}$ cells stimulated in Th1- or Th2-polarizing conditions show the expected profiles of GATA-3 and T-bet mRNA expression. When the cells were stimulated in the absence of exogenous cytokines and blocking antibodies, control T cells ($Itk^{+/-}$) exhibited an interesting pattern of T-bet and GATA-3 expression. Three days after stimulation with a high concentration of the agonist peptide, $Itk^{+/-}$ T cells express substantial amounts of T-bet mRNA but also show significant levels of GATA-3 mRNA. After stimulation with conditions that promote Th2 differentiation, these cells express dramatically high levels of GATA-3, but very little T-bet mRNA, even in cultures that contain mixed populations of IL-4- and IFN-γ-producing cells. In contrast, $Itk^{-/-}$ T cells express moderately high levels of T-bet under all stimulation conditions (Figure 2F). It is particularly striking that $Itk^{-/-}$ cells also express substantial levels of GATA-3 mRNA, suggesting that the low-avidity TCR signals that promote GATA-3 mRNA upregulation are still functional in the absence of Itk (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/21/1/67/DC1). This dual expression of T-bet and GATA-3 may also account for the increased proportion of $Itk^{-/-}$ cells that produce both IFN-γ and IL-4 compared to that seen in $Itk^{+/-}$ cultures (Figures 2C and 2D). Overall, these data indicate that $Itk^{-/-}$ T cells preferentially upregulate T-bet and differentiate into IFN-γ-producing cells under low-avidity TCR stimulation conditions that promote Th2 differentiation in wild-type T cells.

T-bet mRNA Expression Is Aberrantly Regulated by TCR Signals in the Absence of Itk

The regulation of T-bet and GATA-3 expression during T helper cell differentiation is complex, involving TCR signals, cytokine signals, and autocrine feedback loops (reviewed in Murphy and Reiner, 2002). The mechanism by which low levels of T-bet and GATA-3 proteins are activated in naive T cells, as well as how the T-bet and GATA-3 genes are further upregulated following TCR stimulation, has not been fully elucidated. However, our findings suggested that TCR signaling via Itk may be critical for the proper regulation of T-bet and/or GATA-3 levels in stimulated naive $CD4^+$ T cells. To address this possibility, we examined the levels of T-bet and GATA-3 mRNAs, as well as those for IFN-γ and IL-4, in $Itk^{+/-}$ and $Itk^{-/-}$ cells within the first 24 hr after stimulation. In this analysis we examined cells that had been stimulated with $MCC_{93\text{-}103}$ plus APCs in Th1- and Th2-skewing conditions, as well as cells stimulated with the APCs plus the APL, MCC_{T102S}, in both nonskewing (no exogenous cytokine) and neutralizing (with anti-IL-4 and anti-IFN-γ antibodies) conditions. The APL stimulation conditions were specifically chosen for their ability to induce divergent responses from $Itk^{+/-}$ and $Itk^{-/-}$ cells, allowing us an opportunity to determine the underlying molecular mechanism for the aberrant T helper cell polarization of $Itk^{-/-}$ cells in these experiments.

As shown in Figure 3A, naive $Itk^{+/-}$ T cells upregulate T-bet mRNA levels following strong TCR stimulation in both Th1- and Th2-skewing conditions, although within 12 hr after stimulation there is an ~10-fold excess of T-bet mRNA in the cells cultured in IL-12 plus anti-IL-4 antibody. This difference is further enhanced by 24 hr after stimulation, by which time there is a 70-fold increase in T-bet mRNA in the Th1- versus the Th2-skewed cells. GATA-3 mRNA shows an inverse pattern of expression in $Itk^{+/-}$ cells, with a >10-fold increase seen in Th2-skewed cells versus Th1-skewed cells by 12 hr after stimulation (Figure 3B). These patterns of transcription factor expression correlate nicely with cytokine gene expression, as shown in Figures 3C and 3D. IFN-γ transcripts are induced at 12 hr following TCR stimulation in both sets of polarizing conditions, but by 24 hr IFN-γ transcripts have fallen in the Th2-skewed cells and continued to rise in the Th1-skewed cells, resulting in an ~100-fold difference (Figure 3C). Similarly, IL-4 transcripts are just above the level of detection in the Th2-skewed $Itk^{+/-}$ cells at 12 hr and rise dramatically by 24 hr (Figure 3D). Interestingly, the patterns of GATA-3 and IL-4 gene expression are nearly identical between the $Itk^{+/-}$ and $Itk^{-/-}$ T cells stimulated under these conditions (Figures 3B and 3D). In contrast to $Itk^{+/-}$ T cells, $Itk^{-/-}$ T cells show dramatic increases in T-bet mRNA levels at 12 hr after stimulation in both Th1- and Th2-skewing conditions, although the levels of T-bet mRNA do drop substantially by 24 hr in the Th2-skewed cells. In parallel, $Itk^{-/-}$ Th2-skewed T cells also have higher levels of IFN-γ transcripts at the 12 hr time point, which also diminish by 24 hr. One last noteworthy point is that we consistently observe approximately 2-fold higher basal levels of T-bet mRNA in naive $Itk^{-/-}$ T cells, as assessed by quantitative PCR (Figure 3A). This finding has also been verified by microarray analysis of gene expression (C.M. Li and L.J.B.,

neutralizing conditions (anti-IL-4 and anti-IFN-γ antibodies). Cells were stimulated for either 0, 12, or 24 hr. Following the indicated time points, RNA was isolated from cells, transcribed into cDNA, and subjected to real-time quantitative PCR analysis for GATA-3 (A and E), T-bet (B and F), IL-4 (C and G), or IFN-γ (D and H). The y axis values represent the respective transcript normalized to the GAPDH values determined for each sample. The data are graphed on a log scale in (A)–(D) and a linear scale in (E)–(H) to more clearly display the overall magnitude of the changes in gene expression under each set of conditions. Normalization to GAPDH, β-actin, or β2-microglobulin yielded comparable results. Additionally, these housekeeping genes are regulated comparably between $Itk^{+/-}$ and $Itk^{-/-}$ cells (data not shown). Error bars are the SD of transcript values obtained from triplicate reactions. The asterisk next to the horizontal bar indicates the limit of quantitation for the cytokine transcripts. The ratio for the limit of quantitation was calculated by dividing the actual lowest quantifiable copy of IFN-γ or IL-4 cDNA by the average GAPDH value.

unpublished data). Overall these data demonstrate, first of all, that the early Th1 bias in the response of Itk$^{-/-}$ T cells is quickly overturned by the strong cytokine signals provided in vitro with Th2-skewing conditions, indicating that Itk$^{-/-}$ T cells have normal responses to IL-4 signaling pathways. More importantly, however, these data illustrate the aberrant regulation of T-bet and/or IFN-γ transcript levels immediately following TCR stimulation of naive Itk$^{-/-}$ CD4^{+} T cells.

In an effort to address the mechanism by which Itk-deficient cells failed to differentiate into Th2 cells in nonskewing conditions, we examined the levels of T-bet, GATA-3, IFN-γ, and IL-4 mRNAs in response to 100 nM of MCC$_{T102S}$, a condition that induces a mixture of Th1 and Th2 cells in wild-type cultures but only induces Th1 cells in Itk$^{-/-}$ cultures. To exclude the possibility that the initial cytokines produced by the cells could feedback in an autocrine loop and influence gene expression, we performed these stimulations in both nonskewing (no exogenous cytokines) and neutralizing conditions (no cytokines plus anti-IL-4 and anti-IFN-γ antibodies). As shown in Figure 3E, following the stimulation of cells in nonskewing conditions with MCC$_{T102S}$, T-bet expression is rapidly induced in Itk-deficient cells, while it is only weakly upregulated in control Itk$^{+/-}$ cells. In an effort to confirm that T-bet protein levels were also increased in Itk$^{-/-}$ cells, we performed immunoblotting analysis on lysates from control and Itk$^{-/-}$ naive T cells at 16 hr poststimulation but failed to detect any T-bet protein at this early time point. However, consistent with the recent finding that IFN-γ-induced STAT1 signals further upregulate T-bet expression, the presence of neutralizing anti-IFN-γ antibody significantly reduces the T-bet expression in Itk$^{-/-}$ cells by 24 hr (Afkarian et al., 2002). A similar pattern of expression is observed for IFN-γ (Figure 3G). Conversely, GATA-3 transcript levels are rapidly upregulated in both control and Itk$^{-/-}$ cells at 12 and 24 hr poststimulation, although only the Itk$^{+/-}$ cells produce detectable levels of IL-4 transcripts (Figures 3F and 3H). These latter data are consistent with a previous report demonstrating that wild-type and Itk$^{-/-}$ cells regulate GATA-3 comparably (Schaeffer et al., 2001). Taken together, these data clearly demonstrate that, in response to TCR signals, Itk-deficient cells show a dramatic alteration in the regulation of T-bet mRNA expression, but regulate GATA-3 normally.

Collectively, the data presented above indicate that, after stimulation by low-avidity TCR engagement, Itk$^{-/-}$ T cells express increased T-bet mRNA and preferentially differentiate into Th1 cells. This is in direct contrast to Itk$^{+/-}$ T cells, which under these same conditions express very little T-bet mRNA and differentiate predominantly into IL-4-producing cells. Since high-avidity TCR engagement induces Th1 differentiation from both Itk$^{+/-}$ and Itk$^{-/-}$ cells, we were interested in determining whether T-bet transcript levels varied depending on the strength of the TCR signal. As shown in Figure 4, 12 hr following stimulation of naive T cells with a range of MCC$_{93\text{-}103}$ or MCC$_{T102S}$ peptides in the presence of neutralizing antibodies to IL-4 and IFN-γ, Itk$^{+/-}$ T cells show a relatively uniform induction of T-bet mRNA compared to unstimulated cells. In contrast, Itk$^{-/-}$ T cells express increased levels of T-bet mRNA as the strength of the TCR signal decreases. These findings suggest, first of all, that the Th2-prone differentiation behavior of wild-type T cells upon stimulation with low-avidity TCR engagement is not due to a reduced induction of T-bet transcripts, but instead results from increased GATA-3 expression under these conditions (see Supplemental Figure S1). This conclusion is consistent with a previous report demonstrating increased GATA-3 induction in T cells stimulated with low-avidity ligands (Ise et al., 2002). Second, these data suggest that Itk is required for negative regulation of T-bet mRNA expression following low-avidity TCR stimulation.

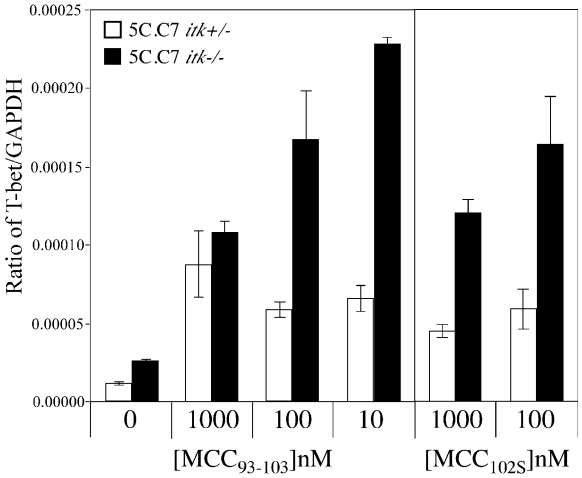


Figure 4. Itk Negatively Regulates T-bet upon Low-Avidity TCR Stimulation

Purified CD4^{+} T cells from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice were stimulated with the indicated concentrations of either MCC$_{93\text{-}103}$ or MCC$_{T102S}$ peptide plus APCs for 0 or 12 hr in neutralizing conditions (with anti-IL-4 and anti-IFN-γ antibodies). Following stimulation, RNA was isolated, transcribed into cDNA, and subjected to real-time quantitative PCR analysis for T-bet and GAPDH. The y axis represents T-bet normalized to the GAPDH values determined for each sample. Error bars are the SD of transcript values obtained from triplicate reactions.

Itk Is Upregulated and Rlk Is Downregulated during Th2 Differentiation

Several years ago, another Tec family tyrosine kinase expressed primarily in T lymphocytes, Rlk/Txk, was identified and was also found to contribute to the activation of PLC-γ, suggesting some functional redundancy within the Tec family (Schneider et al., 2000; Veri et al., 2001). Interestingly, Takeba et al. recently demonstrated that Rlk can function as a transcriptional activator of the IFN-γ gene by binding to the IFN-γ promoter (Schaeffer et al., 1999; Takeba et al., 2002), suggesting a unique function for Rlk in differentiated Th1 cells (Hu et al., 1995; Kashiwakura et al., 1999). In conjunction with these findings, our own data indicating a role for Itk in negatively regulating T-bet expression suggested the possibility that Itk as well as Rlk may be differentially expressed following T cell differentiation. To address this, we analyzed Itk and Rlk mRNA and protein levels in wild-type CD4^{+} T cells differentiating in vitro after stimulation in Th1- versus Th2-skewing conditions. As shown in Figure 5A, we found that Rlk mRNA is rapidly

downregulated within 6 hr following stimulation of naive cells in both Th1 and Th2 conditions. However, Rlk transcripts begin to be upregulated again in Th1 cells after 24 hr and remain increased over the following 11 days of culture, but fail to be reinduced in Th2 cells (Figure 5B). In contrast to Rlk, Itk mRNA levels are maintained in both Th1 and Th2 conditions during the first 24 hr following stimulation (Figure 5C); however, around 8 days poststimulation in Th2 culture conditions, Itk mRNA levels are upregulated (Figure 5D). Interestingly, analysis of the putative promoter regions of Itk and Rlk provide some clues about their differential regulation in Th1 versus Th2 cells. Sequence analysis using the TRANSFAC database (http://www.cbil.upenn.edu/tess) indicated that the promoter region of Itk contains several canonical GATA protein binding motifs, including GATA-3 sites. In contrast, the Rlk promoter region lacks GATA-3 binding motifs, providing a potential explanation for the lack of reexpression of Rlk mRNA in differentiating Th2 cells.

Analysis of Itk and Rlk protein levels in wild-type $CD4^+$ T cells cultured in Th1- or Th2-skewing conditions confirmed the differential expression observed at the mRNA level. As shown in Figure 5E, Rlk protein is detectable in Th1, but not in Th2 cells, whereas Itk protein is present in both cell types, but at a significantly higher level in Th2 cells. Consistent with this latter observation, a recent report found that Itk mRNA is dramatically elevated in T cells from patients afflicted with atopic dermatitis, an inflammatory skin disease in which Th2 cytokines play a major role (Matsumoto et al., 2002). Overall, these findings and the distinct kinetics of Itk versus Rlk regulation during T helper cell differentiation, are summarized in Figure 5F.

Signaling Deficiencies in Th1 versus Th2 Cells Lacking Itk

A number of studies have indicated that there are differences in proximal TCR-induced signaling pathways between Th1 and Th2 effector subsets, including a difference in the organization of their plasma membranes and their usage of lipid rafts (Balamuth et al., 2001, and references therein). To address whether signaling pathways in Th1 and Th2 cells are affected by the differential expression of Itk and Rlk, we assessed the ability of both wild-type and Itk-deficient Th1 and Th2 cells to activate PLC-γ1, as well as the downstream ERK-MAP kinase pathway. Wild-type and $Itk^{-/-}$ cells were stimulated and cultured in Th1- and Th2-skewing conditions for 11 days, a time point at which Itk is upregulated in Th2 cells and Rlk is absent. On day 11, cells were restimulated by anti-CD3 antibody crosslinking, and lysates were prepared for analysis. As shown in Figure 6, both $Itk^{-/-}$ Th1 and Th2 cells show greatly impaired phosphorylation of tyrosine 783 on PLC-γ1, one of the tyrosines that is crucial for the regulation of PLC-γ1 (Kim et al., 1991), compared to the $Itk^{+/-}$ control cells. Interestingly, $Itk^{-/-}$ Th2 cells show a greater deficiency in PLC-γ1 phosphorylation than the $Itk^{-/-}$ Th1 cells.

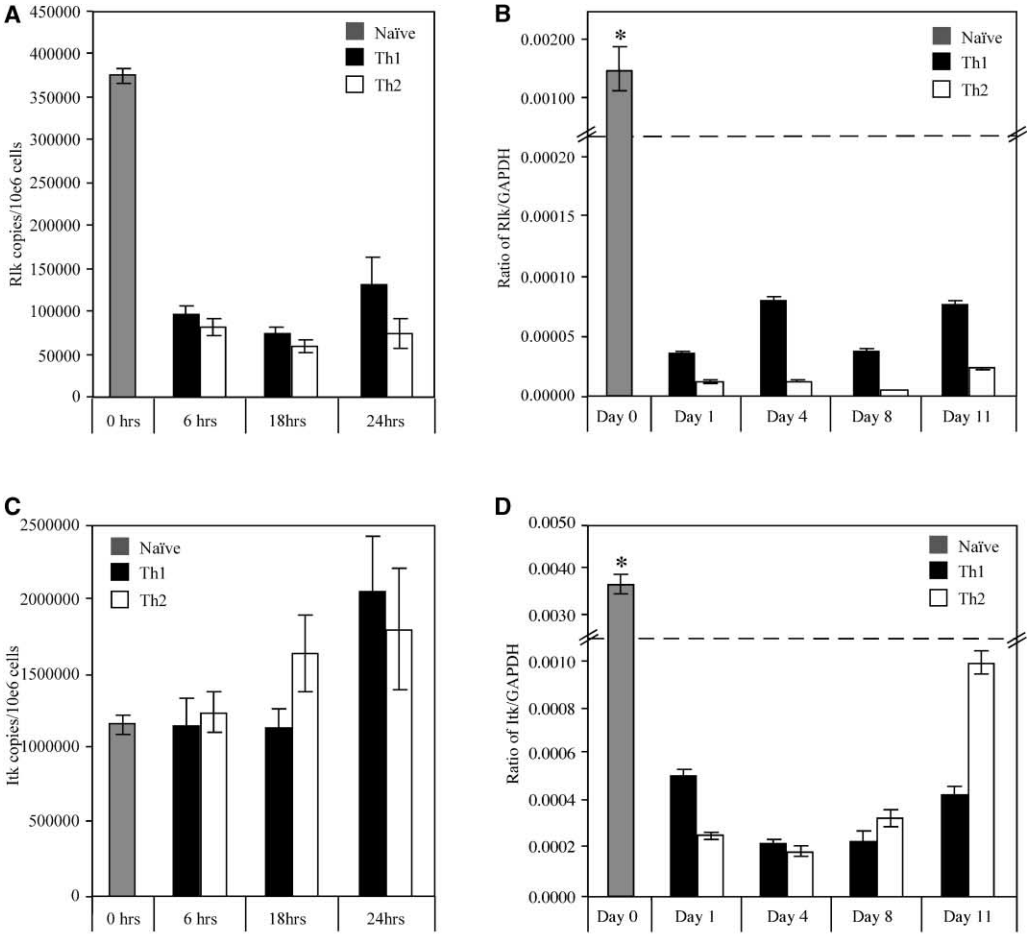
A similar finding was obtained after analysis of ERK activation in Th1 versus Th2 cells lacking Itk. In accordance with impaired PLC-γ1 activation, the phosphorylation of ERK1/ERK2 is more severely impaired in $Itk^{-/-}$ Th2 versus Th1 cells (Figure 6). One potential caveat in this experiment is the observation that $Itk^{+/-}$ Th2 cells show slightly reduced ERK activation compared to the control Th1 cells; thus, Th2 cells, in general, may have a less potent ERK signaling pathway. Nonetheless, following TCR engagement, $Itk^{-/-}$ Th2 cells are more impaired in their ability to activate PLC-γ1 and ERK compared to both control Th1 and Th2 cells, as well as $Itk^{-/-}$ Th1 cells. Collectively, these data support a model in which Itk is not only crucial for the differentiation of $CD4^+$ T cells into Th2 effector cells, but may also be important for the function of differentiated Th2 cells. The upregulation of Itk mRNA and protein in Th2 cells is likely to be critical to compensate for the loss of Rlk from these cells.

Discussion

In this report, we demonstrate that Itk is involved in several aspects of T helper cell differentiation. While previous biochemical studies demonstrated a quantitative deficiency in TCR signaling pathways in the absence of Itk, the data described herein indicate that Itk affects qualitative aspects of the TCR signal as well. We have shown that the activation of Itk following stimulation with low-avidity TCR ligands is a critical factor in promoting Th2 differentiation by negatively regulating T-bet mRNA expression. Thus, these findings have important implications for understanding the mechanism by which different strengths of TCR engagement can lead to distinct fates during T cell differentiation, as well as to the activation of distinct subsets of effector functions.

These data have clarified the mechanism by which Itk influences T helper cell differentiation. Two previous studies established that $Itk^{-/-}$ mice are impaired in their ability to generate a protective Th2 response to pathogens such as *N. brasiliensis* or *S. mansoni* (Fowell et al., 1999; Schaeffer et al., 2001). However, it was not clear whether this resulted from a simple deficit in IL-4 production or was the result of a more complex impairment in Th2 differentiation by $Itk^{-/-}$ $CD4^+$ T cells. Furthermore, the molecular explanation for the observation that Itk-deficient mice preferably generate a Th1 response to pathogens that normally elicit a Th2 response also remained a mystery (Fowell et al., 1999; Schaeffer et al., 2001). Our data have demonstrated that $Itk^{-/-}$ $CD4^+$ T cells are fully capable of differentiating into either Th1 or Th2 effector subsets when exposed to the appropriate cytokines in their environment. But, as previously reported, the resulting Th1 or Th2 effector cells have significant impairments in their ability to produce each subset of effector cytokines (Fowell et al., 1999; Schaeffer et al., 2001). Interestingly, we find that the magnitude of the impairment in cytokine production varies depending on the dose of peptide Ag with which the cells are restimulated, providing a potential explanation for discrepancies between previous reports examining IFN-γ and IL-4 production by $Itk^{-/-}$ T cells (Fowell et al., 1999; Schaeffer et al., 2001).

Our most significant finding is the demonstration that Itk plays a critical role in determining the fate of T cells differentiating in response to low- versus high-avidity TCR signals in the absence of exogenous cytokines.



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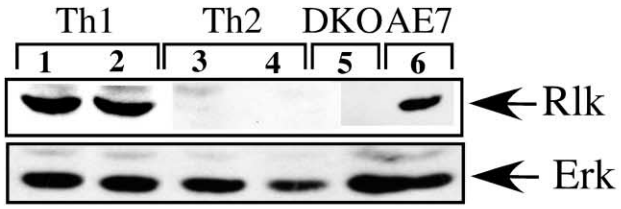

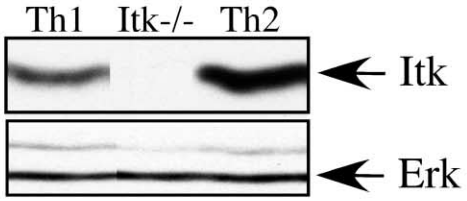

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Th1	Th2	Time	
++	–	<24hrs	Rlk
+++	++++	~8 days	Itk

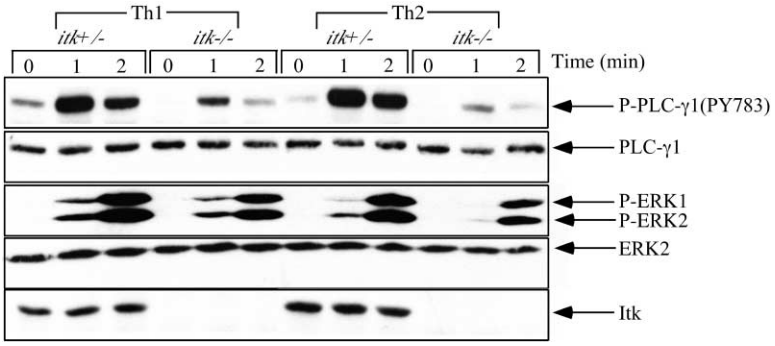

Figure 6. Signaling Deficiencies in Th1 versus Th2 Cells Lacking Itk

Purified CD4$^+$ T cells from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice were stimulated in Th1- or Th2-skewing conditions and cultured for 11 days. Cells were then restimulated by anti-CD3 antibody crosslinking for either 0, 1, or 2 min. Total lysates were immunoblotted with an anti-phospho-specific antibody to PLC-γ1 (P-PLC-γ1(PY783)) or anti-phospho-ERK (p-ERK1 and 2). The membrane was stripped and reprobed for total PLC-γ1, ERK, and Itk protein.

These data provide an interesting contrast to several reports demonstrating that weak TCR signaling induced by low antigen dose, or by low-affinity TCR ligands (APLs), induces transient calcium mobilization and ERK activation, and promotes differentiation into the Th2 lineage (reviewed in Leitenberg and Bottomly, 1999). On the basis of these reports, one might have predicted that Itk$^{-/-}$ CD4$^+$ T cells would be more prone to differentiate into Th2 cells, as several studies have shown that Itk-deficient CD4$^+$ T cells have defects in calcium mobilization and ERK activation following TCR stimulation (Liu et al., 1998; Miller and Berg, 2002a; Schaeffer et al., 1999, 2000). However, the experiments presented here argue that, in the absence of Itk, several aspects of T cell activation and differentiation are altered, the net result of which is to favor Th1 over Th2 differentiation.

The first obvious alteration in Itk$^{-/-}$ CD4$^+$ T cells is their poor production of IL-4, as first described by Fowell and colleagues (Fowell et al., 1999). Our data show that, in contrast to Itk$^{+/-}$ T cells stimulated with an APL, in which IL-4 transcripts can be detected within 24 hr after stimulation, Itk$^{-/-}$ T cells fail to accumulate IL-4 mRNA under these same conditions. This defect is not a result of impaired GATA-3 expression, as Itk$^{+/-}$ and Itk$^{-/-}$ cells have identical levels of GATA-3 mRNA following stimulation with low-avidity TCR signals. Instead, as reported previously, the initial impairment in IL-4 transcription is likely a result of defective NFATc translocation (Fowell et al., 1999) and may be linked to inappropriate modulation of the ratio of NFATc1 and NFATc2 in the nucleus (Brogdon et al., 2002). Interestingly, the defect in Th2 differentiation observed with Itk$^{-/-}$ T cells stimulated in nonskewing conditions can be overcome by the addition of exogenous IL-4, which not only induces GATA-3, thus activating endogenous IL-4 expression, but more importantly, also suppresses Th1 development.

The second, and most striking alteration we observed upon activation of Itk-deficient T cells by low-avidity TCR engagement was the rapid induction of T-bet expression. To date, little is known about the regulation of T-bet gene expression in response to TCR stimulation. The data presented here indicate that Itk signaling functions in a pathway that inhibits this expression. Several recent studies have found increased levels of T-bet mRNA in activated CD4$^+$ T cells lacking either JunB or JunD (Hartenstein et al., 2002; Meixner et al., 2004), suggesting that both Jun factor activities are required to repress T-bet mRNA induction. Consistent with this observation, analysis of the putative T-bet promoter sequence using the TRANSFAC database indicates the presence of several canonical AP-1 binding sites (http://www.cbil.upenn.edu/tess). In addition, activation of JunB in T cells is mediated by JNK (Li et al., 1999), a signaling pathway we have previously shown to be deficient in Itk$^{-/-}$ CD4$^+$ T cells (Miller and Berg, 2002a). These conclusions are also consistent with the data of Schwartzberg and colleagues demonstrating impaired AP-1 activation in T cells lacking Itk (Schaeffer et al., 2001). Thus, one explanation to account for the aberrant expression of T-bet in Itk$^{-/-}$ CD4$^+$ T cells stimulated by low-avidity TCR engagement is that reduced JNK signaling leads to impaired JunB and/or JunD activation and a failure to repress T-bet mRNA induction. Alternatively, our data do not rule out the possibility of a direct affect of Itk on T-bet function that ultimately feeds back to inhibit T-bet expression.

As opposed to the aberrant regulation of T-bet expression in Itk-deficient cells, GATA-3 induction remained

Figure 5. Itk Is Upregulated and Rlk Is Downregulated during Th2 Differentiation

(A–D) Purified CD4$^+$ T cells from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice were stimulated in Th1- or Th2-skewing conditions and harvested at 0, 6, 18, and 24 hr (A and C) as well as at 1, 4, 8, and 11 days poststimulation (B and D). Following the indicated time points, RNA was isolated and subject to real-time quantitative PCR for either Rlk (A and B) or Itk (C and D). (A) and (C) represent Rlk and Itk copy numbers, respectively, normalized to cell number equivalents. (B) and (D) represent Rlk and Itk copy numbers, respectively, normalized to GAPDH values. The asterisk denotes that the indicated ratio is greater in these particular circumstances as a result of GAPDH values being very low in naive T cells compared to activated and expanding cells in culture.

(E) In the top panel, 3.5×10^6 Itk$^{+/-}$ Th1 and Th2 cells were removed from culture at 8 (lanes 1 and 3) and 11 (lanes 2 and 4) days poststimulation. As controls, lysates from Rlk$^{-/-}$Itk$^{-/-}$ (DKO) CD4$^+$ T cells and from the AE7 Th1 cell line were also prepared. Total cell lysates were blotted for Rlk. The filter was then reblotted with antibody to Erk 1/2 as a loading control. In the bottom panel, total cell lysates from 3.5×10^6 Itk$^{+/-}$ Th1, Itk$^{-/-}$ Th2, and Itk$^{+/-}$ Th2 cells at day 10 poststimulation were blotted for Itk. The filter was then reblotted with antibody to Erk 1/2 as a loading control.

(F) A summary of the changes in Rlk and Itk expression levels in differentiating Th1 and Th2 cells. Differences between Th1 and Th2 cells are first observed at 24 hr for Rlk and at $\sim$ day 8 of differentiation for Itk.

largely intact. Several reports have suggested that NF-κB activation via TCR plus CD28 signaling is a crucial event in the induction of GATA-3 transcription but has no bearing on T-bet or IFN-γ expression (Das et al., 2001; Rodriguez-Palmero et al., 1999). In accordance with this finding, Fowell et al. have previously found that NF-κB activation is unaltered in the Itk$^{-/-}$ T cells following TCR/CD28 stimulation (Fowell et al., 1999). Thus, our data showing a role for Itk signaling in the regulation of T-bet, but not GATA-3, expression is consistent with the known pathways modulating these important transcription factors.

Our data also support a model proposing that the strength of TCR signaling influences Th1 versus Th2 differentiation by differential induction of GATA-3 rather than T-bet. Our own data demonstrate that the levels of T-bet mRNA induced within 12 hr in normal CD4$^+$ T cells stimulated with a wide range of antigen concentrations and affinities are remarkably constant. In contrast, we find that GATA-3 mRNA levels increase as the strength of the TCR signal declines (see Supplemental Figure S1), as has previously been reported (Ise et al., 2002).

The data in this report also support the notion that Itk plays an important role in the function of both fully mature Th1 and Th2 lineages. Interestingly, however, we find that Itk mRNA and protein are upregulated during Th2 differentiation, while Rlk, a related Tec kinase, is selectively expressed in Th1 cells and disappears rapidly from Th2 cells. Biochemical experiments revealed that the upregulation of Itk in Th2 cells is an important event that is required for optimal PLC-γ1 and subsequent ERK activation. While our data demonstrate that TCR signaling via Itk is required for the inhibition of T-bet expression during the differentiation of a naive T cell, as discussed above, once a T cell has matured to the Th1 effector lineage, T-bet expression is predominantly dependent on IFN-γ signaling. Furthermore, Mullen and colleagues recently demonstrated that IFN-γ expression in committed Th1 cells is largely independent of T-bet activity (Mullen et al., 2002). Although mature Th1 cells express both T-bet and Itk, TCR signaling via Itk may not actively inhibit T-bet expression in the committed Th1 cell. These data support a model in which Itk is critically required for Th2 differentiation and essential for proper Th1 and Th2 cell function. These findings, together with our own data, suggest a previously unappreciated connection between the Tec family kinases and the factors controlling T helper cell differentiation, GATA-3 and T-bet.

Experimental Procedures

Mice

Itk-deficient mice (Liu et al., 1998), backcrossed to C57BL/10 for over ten generations, were crossed to 5C.C7 TCR transgenic (Jorgensen et al., 1992) RAG$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) to generate H-2^k 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ mice. All mice used were between 6 and 10 weeks old and were maintained in a specific pathogen-free facility.

Abs and Flow Cytometry

Cells were stained with the specified antibodies in HBSS supplemented with 3% FCS for 15 min on ice, washed, and analyzed on a BD Biosciences (San Jose, CA) FACSCalibur. Data were analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). The flow cytometric antibodies used were anti-Vα11-FITC, anti-CD4-CyChrome, anti-CD44-FITC, anti-CD62L-PE, anti-IL-4-PE, anti-IFN-γ-APC (BD Pharmingen).

Preparation and Activation of CD4$^+$ Cells

Spleens and lymph nodes were removed from 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ littermates. Following RBC lysis, spleen and lymph node cells were pooled, and single-cell suspensions were incubated with anti-CD4 magnetic microbeads. CD4$^+$ cells were purified by positive selection (Miltenyi Biotec, Auburn, CA), and were consistently >96% naive (CD44^{lo}, CD62L^{hi}) and expressed the 5C.C7 TCR, which is specific for moth cytochrome C ($MCC_{93\text{-}103}$) (DLIAYLKQATK; Tufts Microchemistry Facility, Medford, MA) and MHC class II IE^k. 1×10^6 purified 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ cells were activated with 1×10^6 mitomycin-C treated (Calbiochem, La Jolla, CA) CH27 cells (B cell lymphoma line, IE^{k+} and B7$^+$) in a variety of different conditions. Cells stimulated in skewing conditions (Th1 = rIL-12 [1 ng/ml] and anti-IL-4 antibody [1 μg/ml]; Th2 = rIL-4 [10 ng/ml] and anti-IFN-γ antibody [0.1 μg/ml] [R&D, Minneapolis, MN]) were activated with 10 nM of $MCC_{93\text{-}103}$ peptide plus CH27 cells. For nonskewing conditions (no exogenous cytokines or antibodies added), cells were stimulated with $MCC_{93\text{-}103}$, MCC_{T102S}, or MCC_{A96S} peptide plus CH27 cells as APCs. After 24 hr, culture media for both skewed and nonskewed conditions were supplemented with rIL-2 (5 ng/ml). Cells were expanded and maintained in their respective culture conditions until restimulation. Itk$^{+/-}$ and Itk$^{-/-}$ T cells expanded comparably in each of the stimulation conditions.

Intracellular Cytokine Staining

3×10^5 T cells were restimulated with 1×10^5 CH27 cells and the indicated concentrations of $MCC_{93\text{-}103}$ for 6 hr in a 96-well plate. Golgi Stop and Golgi Plug (BD Pharmingen) were added for the last 3 hr. The cells were first stained with anti-Vα11-FITC and anti-CD4-CyChrome, fixed, permeabilized, and then stained intracellularly with anti-IL-4-PE and anti-IFN-γ-APC. Cells were immediately analyzed by flow cytometry on a BD FACSCalibur. A minimum of 10,000 CD4$^+$ Vα11$^+$ events were collected.

Cytokine ELISA

5×10^4 T cells that were initially stimulated in Th1- or Th2-skewing conditions were restimulated with 5×10^4 CH27 cells and the indicated concentrations of $MCC_{93\text{-}103}$ for 24 hr in a 96-well plate. Supernatants were serially diluted and assayed for IFN-γ, IL-4, IL-5, and IL-10 using cytokine detection kits (Becton Dickinson).

Real-Time Quantitative PCR

For the analysis of cytokine gene expression during secondary stimulation, 3×10^6 T cells were restimulated with 5×10^5 CH27 cells and the indicated concentrations of $MCC_{93\text{-}103}$ in a 48-well plate for 6 hr. For the analysis of gene expression levels of IFN-γ, IL-4, T-bet, and GATA-3 after the primary stimulation, 4×10^5 purified naive CD4$^+$ T cells were stimulated with 3×10^5 mitomycin C-treated CH27 cells and the indicated concentration of peptide for either 0, 12, or 24 hr in a 96-well plate. For the analysis of Tec family members during T cell differentiation, CD4$^+$ T cells were skewed as above. Following each time point, cells were removed from the plate and total RNA was isolated as previously described (Miller and Berg, 2002a). Primer sequences and reaction conditions used for real-time quantitative PCR are available upon request.

Western Blot Analysis

For the analysis of Tec family protein levels, 3.5×10^6 cells were removed from culture at the indicated time points. The AE7 Th1 clone (gift from Vijay Kuchroo) (Kovac and Schwartz, 1985) and lysates from Rlk$^{-/-}$Itk$^{-/-}$ cells were used as positive and negative controls, respectively. TCR stimulations were done with 5C.C7 Itk$^{+/-}$ RAG$^{-/-}$ and 5C.C7 Itk$^{-/-}$ RAG$^{-/-}$ Th1 and Th2 cells on day 11 following primary stimulation for 0, 1, or 2 min. Lysates were prepared and immunoblotting was performed as previously described (Miller and Berg, 2002a). Antibodies used were anti-Itk (10B2 and 2F12 mAb mix) (Heyeck et al., 1997), anti-Rlk (Santa Cruz), anti-phospho-

783 PLC-γ1 (Biosource International), and anti-phospho-p44/42 MAP kinase (Cell Signaling, Beverly, MA), anti-PLC-γ1 protein (Upstate Biotech, Inc.), anti-p44/42 MAP kinase (Cell Signaling).

Acknowledgments

We thank Luana Atherly, Yoko Kosaka, and Michael Li for their critical review of the manuscript. We also thank Rachel Gerstein (University of Massachusetts Medical Center, Worcester, MA), Ken Murphy (Washington University School of Medicine, St. Louis, MO), Nezih Cereb (Histogenetics, Inc. and Center for Genetic Polymorphisms, Hawthorne, NY), Laurie Glimcher (Harvard School of Public Health, Boston, MA), Pam Schwartzberg (National Institutes of Health, Bethesda, MD), Anjana Rao (Harvard Medical School, Boston, MA), and Mark Bix (University of Washington, Seattle, WA) for plasmids. Last, we thank Martin Felices for technical assistance with the Itk and Rlk Q-PCR experiments. This work was supported by the National Institutes of Health (AI37584). A.T.M. is supported, in part, by a predoctoral training grant awarded to the University of Massachusetts Medical School Graduate Program in Immunology/Virology by the National Institutes of Health (AI-07439).

Received: July 28, 2004
Revised: May 7, 2004
Accepted: May 10, 2004
Published: July 20, 2004

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